Plant biotechnology: a case study of *Bacillus thuringiensis* (*Bt*) and its application to the future of genetic engineered trees

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Abstract: Agricultural productivity may be raised in a sustainable way by many different technologies such as biological fertilizers, soil and water conservation, biodiversity conservation, improved pest control, and changes in land ownership and distribution. Of these measures, biotechnology applications probably hold the most promise in augmenting conventional agricultural productivity, because biotechnology applications give not only the need to increase production, but also protect the environment and conserving natural resources for future generations. Biotechnology applications will have the possibilities to increase productivity and food availability through better agronomic performance of new varieties, including resistance to pests; rapid multiplication of disease-free plants; ability to obtain natural plant products using tissue culture; diagnosis of diseases of plants and livestock; manipulation of reproduction methods increasing the efficiency of breeding; and the provision of incentives for greater participation by the private sector through investments. Insect resistance through the transfer of a gene for resistance from *Bacillus thuringiensis* (*Bt*) is one of the most advanced biotechnology applications already being commercialized in many parts of the world. This paper reviews the development and the status of *Bt* technology and application of *Bt* transgenic plants in current agriculture, and discusses specific issues related to the transfer of the technology to the future of genetic engineered trees with emphasis on conifers.

Key words: Agricultural productivity; *Bacillus thuringiensis;* Genetic engineering; Insect resistance; Trees

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Introduction

One of the most widely-used genetic engineering strategies is the development of Bt-based resistance to insects. "Bt" is short for *Bacillus thuringiensis*, a common soil bacterium that produces an insect toxin. *Bacillus thuringiensis* (*Bt*) was discovered by Ernst Berliner in 1911 (Krattiger 1997). After some more properties of this *Bacillus* species had been identified, it did not take long until first experiments were being carried out on its effectiveness in controlling the corn borer (Krattiger 1997). When first problems with synthetic pesticides turned up, serious attempts were made to establish *B. thuringiensis* as a biological pesticide (Krieg 1986). In the last few years, several crops have been genetically engineered to produce their own Bt toxins, making them resistant to specific groups of insects. Genetically engineered (GE) plants with Bt-based insect resistance produce an insect toxin in all of their tissues. *Bacillus thuringiensis* bacteria produce proteins called delta-endotoxins that are toxic to insects when ingested. When an insect consumes the protein, protease enzymes in the insect's digestive system cut the normally non-toxic protein into a smaller piece that is highly toxic to insects. The smaller, activated form of the delta-endotoxin binds to a specific receptor on the surface of cells lining the insect's gut, causing a disruption of electrolyte balance, leading to death. In 1995 the market volume of Bt preparations was at an estimated 90 million US dollars and 67 preparations were registered worldwide (Kumar *et al.* 1997). Forecasts made in 1991 predicted that by the year 2000 Bt preparations would account for 5-10% of the world insecticide market (Bernhard and Utz 1993). The main target pests of Bt insecticides include various lepidopterous species such as butterfly, dipterous species such as flies and mosquitoes, and individual coleopterous species such as beatle. Some strains have also been found to kill off nematodes (Edward *et al.*; 1988; Krieg and Franz 1989). Conventional Bt preparations registered in worldwide are mostly derived from the highly potent strain *Bacillus thurigiensis* var. kurstaki HD1, which was isolated in the sixties (Kumar *et al.* 1997). Bt-toxins have been considered very safe for human consumption because the intestinal walls of mammals do not have the endotoxin receptor necessary for the toxic effect, and the proteins are degraded quickly in the stomach. There are actually many variants of Bt-toxins found in nature. One of the unique features of this family of insect toxins is that different toxins affect different groups of insects. For example, the Bt-toxin most commonly used in genetic engineering, named *Crylab*, kills only moths and butterflies (Lepidoptera), but not insects in other insect families. The various delta-endotoxins are given names that begin with "*Cry*" because the endotoxins normally exist in a crystalline form. All Bt-plants to date have only one

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version of endotoxin each. Eventually, combining multiple versions of Bt-toxins in the same plant could provide resistance to several different families of insects at the same time. This article review: 1) Production of Bt transgenic plants; 2) Field trials of transgenic plants with Bt genes; 3) Commercialization of Bt transgenic plants; 4) Impact of Bt transgenic plants on environment; 5) Genetic engineered trees with Bt genes.

Bt transgenic plants

Many researchers have hailed transgenic insecticidal crops-plants modified to produce insecticidal proteins derived from genes of the bacterium *Bacillus thuringiensis*-as the most important technological advancement in insect pest management since the development of synthetic insecticides (Perlak *et al.* 1991). At least 18 transgenic insecticidal crops have been field-tested in the United States, and three (corn, cotton, and potato) have been widely planted (Andow and Hutchison 1998; Federici 1998; Gould and Tabashnik 1998). As the commercial availability of these crops has grown, controversy over how to assess and manage the risks has posed by this method of pest control. Several important crops have been engineered to express toxins of *Bacillus thuringiensis* (*Bt*) for insect control. In 1999, US farmers planted nearly 8 million hm^2 of transgenic *Bt* crops approved by the US Environmental Protection Agency (EPA). *Bt*-transgenic plants can greatly reduce the use of broader spectrum insecticides. Present resistance management strategies rely on a "refuge" composed of non-*Bt* plants to conserve susceptible alleles. The field tests also examined the strategy of spraying the refuge to prevent economic loss to the crop while maintaining susceptible alleles in the population. Each insect/*Bt* crop system may have unique management requirements because of the biology of the insect, it is important to also develop the next generation of technology and implementation strategies. At present, insect assays of Bt transgenic plants demonstrated that at least 32 insect species can be controlled by delta-endotoxins (Table 1). The widespread planting of millions of hectares of transgenic crops with high levels of insecticidal proteins raises concerns that pest populations might develop resistance to Bt toxins and that food webs might be disrupted (Gould 1991). Indeed, the US Environmental Protection Agency (EPA) requires industry to maintain populations of susceptible (nonresistant) insect pests to slow development of resistant populations. Nor are concerns limited to the United States: Anxiety over the safety of food and products derived from transgenic crops has created tensions among international trading partners (Balter 1997).

Citing statistics showed that two billion people worldwide suffer from malnutrition and vitamin deficiencies. Much of this human suffering can be alleviated in a sustainable manner if we were to (1) increase funding for plant genomics and plant biotechnology research to increase yields, enhance micronutrients in food, and create edible vaccines, and (2) if we were to target funding for the training of scientists and plant breeders from developing countries in biotechnology. Current methods of delivering vaccines and vitamins to those in developing countries that require ongoing, annual expenditures from the industrialized world (Brower 1996; Butler and Reichhardt 1999).

Table 1 List of latin names for cited insect species

English names	Latin names
Beet armyworm	*Spodoptera exigua*
Cabbage caterpillar	*Pieris rapae*
Cabbage looper	*Trichoplusia ni*
Cabbage worm	*Pieris brassicae*
Cabbage caterpillar	*Pieris rapae*
Cabbage looper	*Trichoplusia ni*
Cabbage worm	*Pieris brassicae*
Codling moth	*Cydia pomonella*
Colorado potato beetle	*Leptinotarsa decemlineata*
Corn Earworm	*Helicoverpa zea*
Cotton bollworm	*Heliothis, Pectinophora, Earias*
Cotton leaf perforator	*Bucculatrix thurberiella*
Cotton leaf worm	*Spodoptera littoralis*
Cupreous chaffer beetle.	*Scarabaeid* spp
Diamondback moth	*Plutella xylostella*
European corn borer	*Ostrinia nubilalis*
Greater wax moth	*Galleria mellonella*
Green lacewing	*Chrysopa carnea*
Gypsy moth	*Lymantria dispar*
Hessian fly	*Mayetiola destructor*
Honey bee	*Apis mellifera*
Indian meal moth	*Plodia interpunctella*
Ladybird beetle	*Hippodamia convergens*
Mosquitoes	*Aedes aegyti, Culex pipiens*
Navel orangeworm	*Amyelois transitella*
Parasitic wasp	*Nasonia vitripennis*
Pink bollworm	*Pectinophora gossypiella*
Porina moth	*Wiseana* spp.
Saltmarsh caterpillar	*Estigmene acrea*
Silk worm	*Bombyx mori*
Spotted cucumber beetle	*Diabrotica undecimpunctata*
Stemborer	*Scirpophaga incertulas*

Plant biotechnology has made significant advances in delivering the Hepatitis B vaccine in corn and bananas and the cholera vaccine in potatoes. Plant biotechnology also has other benefits, such as the development of crops that can endure droughts, pests and plant diseases that can devastate crops, leading often to population-wide starvation. When scientists solve these problems in poor countries, they not only help people feed themselve and move up from poverty, but also help ensure safe harvests. Most Bt preparations available on the market, all of which are subject to individual licensing, contain spores with parasporal inclusion bodies composed of δ-endotoxins. To date, different δ-endotoxin genes have been isolated which

fall into sixteen subgroups (Crickmore *et al.* 1997). These genes can occur in different strains and in diverse combinations. Almost all Bt strains are able to form more than one type of crystalline inclusion body, and these in turn can be made up of several different δ-endotoxin molecule species. Bt strains can also exchange plasmids containing δ-endotoxin genes and so express different activity patterns in different lepidopterous species. The spores and crystalline toxin molecules are inactivated quickly when exposed to UV-light (Munkvold *et al.* 1999). The final toxic fragment of the most frequently encountered protein, *CrylA*, is thought to span amino acids 29-608, counting from the Nterminus (Hofte and Whiteley 1989). Toxin protein binds to specific receptors located in the insect gut. According to present knowledge this leads to the formation of pores and consequent destruction of ion gradients. These pores also permit the vegetative Bt cells germinating from the spores to migrate into the haemolymph and promote the intoxication process through the ensuing bacteraemia (Marrone and MacIntosh 1993). This suggests that the insecticidal action of Bt must consist of highly complex interactions between the bacterium and its individual host insect species (Asano and Hori 1995).

On the pathogen side we have a great diversity of toxin genes that can occur in varying combinations within a strain and are capable of exchange between strains via conjugation-like processes. By virtue of their variable and conserved regions and occasional flanking transposon sequences Bt toxin genes are predestined for multiple transposition and recombination, suggesting that the great variability of Bt is also attributable to the individual structure of any given toxin gene. The insecticidal action of Bt is enhanced by a concurrent induction of bacteraemia following the binding of toxin proteins to and resultant formation of pores in the intestinal wall, as well as by the recently discovered growth factors. Two prerequisites for infection on the host side are that the intestinal environment must permit efficient solubilisation of the crystalline inclusion bodies and that the proteases the host cleave the solubilised protoxins such that the resulting active toxins are of the right size. This is essential if the proteins are to diffuse through the peritrophic membrane and reach their specific receptors in the instestinal wall. It is thus evident that insects have developed both specific and unspecific defences acting at different levels to protect them against the insecticidal action of Bt upon ingestion of parasporal inclusion bodies. Receptor assumes a key role in this defence. The absence of the necessity to solubilize the endotoxin molecules and to cleave down the protein to a smaller size in insects feeding on transgenic plants may have an influence on the susceptibility of non-target organisms. To date there have only been few reports of initially susceptible insect species evolving resistance after intensive use of Bt preparations in the field. Some of the studies used purified toxins and insofar mimic the situation for transgenic plants but not for the conventional Bt preparations (Marrone and MacIntosh 1993). The fact that resistance development in the open field with conventional Bt-preparations is very rare up to now sheds light on the significance of the differences between transgenic insect resistant plants and the commercially available Bt-preparations. It is surprising that the discussion on the possibility of resistance evolving in initially susceptible insects as a result of the use of transgenic plants has given so little attention to these aspects.

Transgenic plants with Bt genes have been produced in several countries (Table 2).

Table 2. Transgenic plants with Bt genes

Institution	Country	Major Bt Research	Major Pest	Major Crops
CSIRO	Australia	Bt expression	*Lepidoptera*	Cotton
University of Ottawa	Canada	*CrylA(b, CrylA(c))*	*Lepidoptera*	Maize
Beijing University	China	Bt transgenic plants	*Lepidoptera*	Solanaceae
Central China Normal University	China	*CrylA*	Cabbage caterpillar	Rutabaga
National Plant Genetics Laboratory	China	*CrylA(c)* gene	*Lepidoptera*	Cabbage
Osmania University	India	Transformation	*Heliothis sp.*	Cotton
Tokyo University	Japan	Bt ICP gene	*Lepidopteran*	*Azospirillum*
Horticultural research	New Zealand	Transgenic clover	Porina moth	White clover
Wageningen University	Netherlands	*Cry1A(b)* and *Cry1C*	*S. exigua, Maduca sexta*	Tobacco, tomato
Russian Academy of Sciences	Russia	Bt *CrylA(c)*	Colorado potato beetle	Potato
Federal Institute of Technology	Switzerland	*CrylA(c)*	*Lepidoptera*	Rice
Georgia University	USA	*Cry1A(c)*	*Lepidoptera*	Peanut, Soybean
Michigan State University	USA	Bt *(Cry IA(c)*	*Lepidoptera*	Potatoes, Juneberry
Georgia University	USA	*CrylA(c)*	Budworm,	Tobacco
New Jersey State University	USA	*CrylIIB*	Colorado potato beetle	Eggplant
Ohio State University	USA	Bt cp genes	*Lepidoptera*	Sweet gum
Oregon State University	USA	Bt ICP genes Poplar	*Lepidoptera*	Poplar, Potato
Cornell University	USA	*CrylA*	Diamond back moth	Broccoli, cabbage
University of California	USA	*Cryla(c)*	Codling moth	Walnut, strawberries

Bt corn is a very good model system to examine resistance management strategies. Over 2.8 million hm^2 of Bt corn were planted in the United States in 1998, limited only by seed availability (Andow and Hutchison 1998); about 8 million hm^2 of Bt corn were planted in 1999. Thus, although acreage declined to approximately 6.2 million hm^2 in 2000, Bt corn is now the most common management tactic for the European corn borer, Ostrinia nubilalis, throughout the corn-growing regions of the United States. The potential benefits of transgenic insecticidal corn include savings in resources devoted to scouting for pest insects, reduced applications of broad-spectrum insecticides, increased or protected yields due to season-long control of *O. nubilalis* (Rice *et al* 1995), protection of stored corn from lepidopteran insect pests, and lower mycotoxin levels due to a reduction in fungal plant pathogens associated with *O. nubilalis* feeding (Munkvold *et al.* 1997). Balanced against these potential benefits are possible drawbacks. Such disadvantages of genetically modified crops can be grouped into three categories: (1) selection for resistance among populations of the target pest, (2) exchange of genetic material between the transgenic crop and related plant species, and (3) Bt crops' impact on nontarget species. The potential for *O. nubilalis* to develop resistance to toxins in Bt corn has been discussed in several publications (Gould *et al.* 1997). Although the transfer of genetic material between Bt corn and its wild relatives can be a concern (Bergelson *et al.* 1998), the potential for that transfer is limited to Mexico and Central America, where the wild species are located (Galinat 1988). Recent studies documenting negative impacts indicate that nontarget effects may be subtle and complex, and thus may be overlooked in the risk assessment conducted during the registration process for governmental approval of this transgenic crop.

Field trials of transgenic plants with Bt genes

The field experiment examined the effect of refuge size and refuge placement on the distribution of the larvae within the plots as well as the level of resistance in diamondback moths at the end of the season. The results demonstrated that the cumulative number of larvae per plant on refuge plants through the season in the 20% mixed refuge was significantly lower than the 20% separate refuge. This finding indicates that a separate refuge is more effective at conserving the number of susceptible alleles because larvae on these refuge plants will be more likely to survive to adults that can mate with resistant individuals and thereby reduce the number of resistant offspring. This finding provides evidence to support the use of a separate refuge for *Bt*-transgenic crops that are attacked by insects that can move between plants as larvae. Comparing the level of resistance at the beginning of the test to the level at the end, it appears that the insects actually became more susceptible (Sedlacek *et al.* 2001). The results from field study might be taken as justification for not needing any refuge within a planting because of the presence of immigrating susceptible alleles. However, such an approach would only be justified if immigration patterns of susceptible insects were well known and had been shown to be consistent. Usually one does not know a priori whether such immigration of susceptible alleles will occur. Thus, current recommendations allow the management of insects on these refuge plants through the use of insecticides with a different mode of action than the *Bt*-transgenic plants. The critical question in such a strategy is whether enough susceptible insects will survive in the refuge to provide an effective source of susceptible alleles (Giles *et al.* 2000). Insects collected from the *Bt* plants would have a resistant genotype for *Bt* var. *kurstaki* resistance, and we consistently found significantly higher numbers of *Bt* var. *kurstaki*-resistant larvae on the *Bt* plants when the refuge was sprayed than when it was not sprayed. This is the opposite of what should occur if resistant alleles are to be maintained in the refuge for an effective resistance management strategy (Glare and O'Callaghan 2000). Within an individual field or farm, treating the refuge with a highly effective insecticide may dilute the abundance of susceptible alleles to such an extent that the refuge is rendered ineffective unless there is substantial immigration of susceptible alleles from wild hosts or from surrounding non-*Bt* crops (Fearing *et al.* 1997). On the other hand, growers may be reluctant to sacrifice a large number of refuge plants to insects just to maintain susceptible alleles (Croft 1990). An alternative to the strategy of having a 20% refuge that can be sprayed is the EPA-approved strategy of having a 4% refuge that remains unsprayed. Critical experiments need to be performed to assess which approach, as well as which refuge size, would be more effective in conserving susceptible alleles while providing acceptable crop yields, and such tests need to be performed in the specific insect/*Bt* crop system.

As the resistance management strategies are refined for the currently available *Bt* crops, it is also imperative that other strategies for managing overall resistance to *Bt* be developed and implemented in the near future (Archer *et al* 2000). Currently the most promising ones being evaluated in transgenic plants include vegetative insecticidal proteins, as well as various genes from other insects, animals, plants, and bacteria that act as inhibitors of insect digestive enzymes. The development and implementation of engineered insecticidal plants is currently in its infancy and the only available technology is that of *Bt*-transgenic plants. Although the diamondback moth/*Bt* broccoli system may not exactly duplicate the currently available insect/*Bt* crop systems such as cotton, corn, and potatoes, it can help identify areas for further work. Concurrently with more field studies conducted to refine the presently utilized recommendations, industry, public sector scientists, and farmers must work together to develop a second generation of technology and implementation strategies to ensure the even longer term durability of *Bt*-transgenic plants (Barry

and Darrah 1991; Bhowmik 1994). Using field-collected diamondback moth populations and laboratory susceptible population scientists were able to make synthetic populations of diamondback moth with the desired resistance allele frequency for Cry1A toxins. Although these synthetic populations were manipulated for field studies in isolated research plots, there was no carryover through the winter, since the diamondback moth does not overwinter in field where these tests were conducted. Plots with a separate refuge had two border rows on one side of the field, and the border rows were separated from the *Bt* plants by one blank row of bare ground. Mixed refuges had non-*Bt* plants randomly assigned within the plot. Insect bioassays with *Bt* var. *kurstaki* (Novartis) to evaluate resistance were done with progeny of the released larvae and with progeny of larvae counted in the final collection. At present, field trails of transgenic plants with Bt gene are well done most in corporations (Table 3).

Table 3. List of Major Corporations Developing Transgenic Crops with *Bt* Genes

Company	*Bt* gene	Major Focus	Field trials
AgrEvo	*cryIA(b)*	Potato, Corn	Small scale
American Cyanamid	*cryIA(c)*	Cotton	Small scale
Cargill	*CBI-Bt*	Corn	Small scale
DeKalb Genetics	*cryIA(b)*	Corn	Large scale
Delta and Pine Land)	*cryIA(c)*	Cotton	Commercialized
DowElanco	*cryIA(c)*	Corn	Small scale
ELM/Asgrow	CBI-*Bt*	Corn	Small scale
Frito Lay	*cryIIIA(a)*	Potato	Small scale
Genetic Enterprises	CBI-*Bt*	Corn	Small scale
Hunt Wesson	*cryIA(b)*	Corn	Small scale
Miles	*cryIA(c)*	Cotton	Small scale
Monsanto	*cryIA(b)*, *cryIA(c)*	Corn, Cotton, Potato	Commercialized
Mycogen	*cryIA(b)*	Corn	Commercialized
Novartis	*cryIA(b)*	Cotton, Corn Tomato,	Commercialized
Pioneer Hi-Bred International	*cryIA(b)*	Corn	Large scale
Rohm and Haas	*cryIA(b)*	Tobacco	Small scale

Commercialization of Bt transgenic plants

As insect resistant transgenic plants are engineered by means of single isolated toxin genes, the fact that the genetic code of plants differs slightly from that of bacteria makes it necessary to use synthetic genes whose nucleotide sequence is altered in such a way that it still encodes the desired bacterial amino acid sequence (Malik 1997). The insect resistant maize engineered by Novartis contains two copies of a truncated synthetic *CryIA (b)* gene. This gene comprises the first 648 amino acids of a protoxin that is normally made up of 1155 amino acids (Tabashnik 1994). The 648-residue protein probably has to undergo two or three more proteolytic steps in an alkaline environment before it becomes the 564-578-residue protein that Novartis declares to be the fully active toxin (Lereclus *et al.* 1993; Perlak *et al.* 1991). It is probable that the solubilised toxin molecule can diffuse directly through the peritrophic membrane without further cleavage steps (Tabashnik et al 1997). Expression of proteins produced by a common bacterium, *Bacillus thuringiensis* (*Bt*), in transgenic plants to protect them from insect attack is revolutionizing agriculture (Roush and Shelton 1997). The insecticidal proteins produced by *Bt* are toxic to major pests of many of the world's most important crops such as cotton, rice, and corn. Of the $US 8.1 billion spent annually on insecticides worldwide, it is estimated that nearly $2.7 billion could be substituted with *Bt* biotechnology applications (Krattiger 1997). At least 16 companies are presently developing transgenic crops with *Bt* genes, and at least 18 *Bt*-transgenic crops have been approved by the US Department of Agriculture (USDA) for field testing and their research results were patented (Table 4).

Table 4. Institutions Holding *Bt*-Related Patents

Institution	Country	Total
Australian National University	Australia	3
CSIRO	Australia	3
National Research Council	Canada	2
Institut Pasteur	France	11
AgrEvo	Germany	22
BASF	Germany	3
Agrartudomanyi-Egetem	Hungary	2
Agency for Industrial Science	Japan	2
Mitsubishi	Japan	3
Nissan Chemical	Japan	3
Fukuoka-Ken	Japan	4
Korea Chem	Korea	2
Wageningen University	Netherlands	2
Kamenek L K	Russia	2
State Research Institutes	Russia	18
Novartis	Switzerland	33
Zeneca	UK	13
Agracetus	USA	3
Abbott-Laboratories	USA	27
Biotechnica International	USA	2
Cetus	USA	4
Drexel University	USA	2
DuPont	USA	3
Ecogen	USA	19
Lubrizol-Genetics	USA	9
Monsanto	USA	17
Mycogen	USA	81
Pioneer Hi-Bred International	USA	2
University of California	USA	4
University of Wyoming	USA	2
Washington Research Foundation	USA	3

When incorporated into plants, *Bt* proteins are made much more persistent and effective, even against insects that feed at sites difficult or impossible to reach with sprays.

Bt cotton was one of the first insecticidal plants to be approved for commercial use in 1995, and since then the adoption of this technology has been rapid not only in the United States but also in Australia and China. The reasons for the rapid adoption of this new technology are compelling. Despite the considerable advantages of *Bt*-transgenic crops, both to the environment and to farmworker safety, concern is widespread that these gains will be short-lived because of evolution of resistance in the pests. Various deployment strategies have been proposed to delay the onset of resistance (McGaugher and Whalon 1992), and modeling studies have examined the effect of different deployment strategies (Alstad and Andow 1995; Tabashnik 1994); however, few empirical data exist. The refuge is composed of nontransgenic plants that will generate enough SS (homozygous susceptible) individuals to outnumber RR (homozygous resistant) individuals during mating, so that the majority of the population will remain either RS or SS.

Recently the debate on the appropriate strategies for controlling insects through the use of *Bt* plants has focused on the size of the refuge needed (Hargrove 1999), or indeed whether refuges that are large enough can be economically acceptable to the users or sellers of *Bt* crops. In cotton, for example, some workers have called for a dramatic increase in refuge sizes over the Environmental Protection Agency (EPA) requirements, such as refuges as large as 50%, if farmers are allowed to spray them (Gould and Tabashnik 1998). The use of current transgenic cultivars thus faces the following dilemma. The maximum benefits to crop production, farm profitability, and reduction of pesticide use may come from larger proportions of transgenic crops, but long-term enjoyment of these benefits may be feasible only by limiting the percentage of the crops that are transgenic. Careful modeling studies and empirical data are needed to address this question. Testing a resistance management strategy is inherently difficult because it requires both a *Bt*-expressing plant and an insect that has developed resistance to the *Bt* toxin expressed in the plant (Shelton 1993). Resistance in this population of diamondback moth was due to a single autosomal recessive gene, and the plants expressed high levels of the toxin (Metz *et al.* 1995). The pure stands of *Bt*-expressing plants resulted in rapid development of highly resistant diamondback moth populations, and increasing the size of the refuge delayed the development of resistance. Furthermore, the placement of the refuge plants significantly affected the development of resistance. When both plant types were mixed in a random spatial arrangement, larvae were able to move between plant types. As they moved from refuge plants to *Bt*-expressing plants, they died and caused an overall decline in the number of susceptible alleles. This resulted in a more rapid development of resistance than when plants were separated by a distance that limited the movement of larvae. Additional greenhouse and laboratory data demonstrated that resistant diamondback moths display similar levels of weight gain, growth, and survival on *Bt* plants as they do on non-*Bt* plants (Tang *et al.* 1999). These studies have documented that *Bt*-resistant insects can survive on *Bt* plants and that different management strategies will influence the durability of resistance. Although these studies provided some insight into variables that could be manipulated to delay the onset of resistance, the present field study was performed to provide further data to help identify variables that may influence resistance management in the field.

Impact of Bt transgenic plants on environment

Because research has shown that microbial insecticide formulations of Bt have some negative effects on natural enemy species (Rough 1997), it is important to determine the impact of Bt corn on populations of insect predators and parasitoids in the crop ecosystem (Talekar and Shelton 1993). Transgenic crop affects natural enemies in several ways. The enemy species may feed directly on plant tissues or host populations may be reduced. Data submitted for governmental registration of transgenic crops appear to focus primarily on direct feeding on corn tissues (Abbas *et al.* 1995; Armstrong *et al.* 1995). However, increased mortality of lacewing (*Chrysoperla carnea*) larvae was observed when the larvae fed on an artificial diet containing Bt toxin or preyed on corn borers or other lepidopteran larvae that had fed on transgenic corn (Davis and Coleman 1997). Indirect negative effects on predators have not been documented in the field; sampling from transgenic cornfields has not shown declines in predator abundance. If Lepidoptera and their predators and parasitoids are significantly reduced in Bt crop fields and adjacent margins, we might expect the insect prey available for birds, rodents, and amphibians to decrease (Cotty *et al.* 1997). When Bt sprays were purposely used to reduce caterpillar abundance in a forest, fewer black-throated blue warbler nests were observed in sprayed areas. Therefore, it is necessary to access the risks from Bt crop based solely on toxicological studies that examine direct effects of Bt toxins on potential nontarget organisms.

The foundation for regulation of transgenic Bt crops is based on a history of relatively safe use of Bt sprays (Koziel *et al.* 1993). The rapid break-down of Bt toxins in the environment reduces the effects on nontarget organisms, although studies of the ecological interactions of Bt insecticide sprays have documented some effects on nontarget organisms (Mason *et al.* 1996). The lepidopteran species most likely to be affected by Bt corn pollen can be determined by examining their distribution and phenology (Kumer *et al.* 1997). Plant communities within range of corn pollen dispersal will, to a large degree, determine which herbivore species are most likely to be present and subject to the effects of Bt corn pollen. An initial list of nontarget lepidopteran species can be generated by cross-referencing the species of plants likely to be found near

corn with the species of Lepidoptera that feed on these and related plant species. Because many plant species in and around cornfields are considered to be weeds, the makeup of these plant communities is fairly well known (Malik 1997). Although the toxin in Bt crop is active against several lepidopteran families, variation in susceptibility has been observed (Roush and Shelton 1997). It may be possible to link susceptibility and phylogeny to allow prediction of susceptibility of a given lepidopteran species. Integrating distribution, phenology, and susceptibility permits a ranking of the risk to specific lepidopteran species. Species at particularly high risk could then be identified for further testing.

Based on these laboratory and field results, it appears that pollen from Bt crop may pose a risk to monarch populations. Monarchs may also be negatively affected by the use of transgenic corn and soybeans that are resistant to the herbicides Roundup and Liberty. If these herbicides are used to kill weeds in these transgenic crops, then the abundance of milk-weed, which supports monarch populations in agricultural fields, will decline. Considering the seasonal life cycle of insects, there are potentially important differences between the use of soil insecticides at planting and the occurrence of transgenic Bt toxins in roots (Federici 1998), pollen deposition, and stalk residues at harvest. An ecological approach to evaluating the effects of Bt crop would greatly enhance the effectiveness of the registration process in assessing the potential nontarget effects of this new technology (Munkvold *et al.* 1999). Plant pathogens also produce mycotoxins, which may be harmful to humans and livestock; levels of these compounds also were reduced in transgenic corn (Munkvold et al. 1999). Two strategies for reducing risks for nontarget species associated with pollen from Bt crop. The simplest strategy would be to use only those Bt-crop hybrids that do not express the Bt toxin in the pollen. Expression of genes in pollen was controlled by single gene promote, and there are commercial hybrids that do not express detectable levels of Bt toxins in pollen. This lower efficacy may present problems for resistance-management programs that are based on high mortality of target populations. The size and shape of the areas of non-Bt crop would have to be designed carefully to ensure that they effectively serve both purposes. The second is simply not planting transgenic crop hybrids and this would eliminate the potential risks to nontarget species that this biotechnology poses.

Genetic engineered trees with Bt genes

Forest biotechnology has developed very fast in the last 15 years. Many molecular techniques have been established to analyze tree genomes as well as transfer of genes into their genomes. To improve trees via genetic engineering, genes must be available which code for appropriate tree-specific characteristics like tree growth or wood quality parameters such as wood density, lignin and cellulose content. Another prerequisite for genetic engineering of plants is the establishment of an efficient *in vitro* culture system to regenerate plants from single cells (Tang *et al.* 2001). Nowadays, many genes and regeneration methods are available which can be used in transformation experiments of trees. Without doubt by using the novel methods forest tree breeding will speed up at an accelerated level (Griffin 1996, Fladung *et al.* 1997; Tzfira *et al.* 1998). Many gymnosperm (conifers) and angiosperm tree species have been considered so far for improvement via genetic engineering. Scientists at Union Camp, Westvaco, and other paper companies are engineering sweet gum and cottonwood and hoping to create a super tree one that grows faster than normal but retains hardiness. Other scientists have had more success engineering trees to control weeds and insects that plague tree plantations. The Oregon State University Tree genetic Engineering Research Cooperative, a consortium of companies, government agencies, and universities, has engineered hybrid poplars to resist the herbicide glyphosate and produce insecticidal Bt toxins. Glyphosate, which is toxic to ordinary poplars, is not used in growing trees except to clear sites. However, with glyphosate-resistant trees, growers could spray plantations with glyphosate. This research program has also produced transgenic hybrid poplars to produce Bt toxin, in the hopes of controlling a serious leaf-eating pest, the cottonwood leaf beetle. California and Swedish scientists are trying to speed up traditional tree breeding by transferring a gene that accelerates flower development from Arabidopsis into the European aspen. Typically, an aspen is 10 to 20 years old before it produces flowers, which are essential for traditional breeding crosses. With the gene from Arabiodopsis, scientists hope to produce aspens that begin flowering at a much younger age. While no permits for commercialization have been requested for transgenic trees, the above information demonstrates that several different types of transgenic trees have been developed and are currently in field trials. It is anticipated that the first transgenic trees will start appearing on the open market in 3 to 5 years.

Two methods, namely the *Agrobacterium tumefaciens* system and the particle gun bombardment are currently well established to introduce foreign genes into forest tree genomes. The easiest and best known method to transform many angiosperm and few gymnosperm species is the transfer of genes via *Agrobacterium* (Jouanin *et al.* 1993). This method is cheap, easy to handle and produces transgenic lines carrying only one to few copies of the transferred gene. In those angiosperm species which are not susceptible to *Agrobacterium* infection the particle gun bombardment is an alternative method to transfer relevant genes into the host genomes (Tzfira *et al.* 1998; Jouanin *et al.* 1993). For many conifer species particle gun bombardment is the most successful transformation method so far (Jouanin *et al.* 1993; Walter *et al.* 1998). The disadvantages of the bombardment method are that the conditions for shooting the DNA-coated gold particles have to be

newly established for each species, and the transgenic trees often carry multiple copies of the transferred genes and/or truncated fragments of the gene construct. However, reports have been published which describe the application of the *A. tumefaciens* system also in conifer species (Stomp *et al.* 1990; Huang 1991; Tzfira *et al.* 1996). Many genes involved in lignin biosynthesis have been cloned and used for transformation of tree species in the last five years (Boudet 1998). The interest of the industry is to reduce the costs and minimise the ecological demands when using highly reactive chemicals in the process of removing the lignin. Attempts are underway to reduce lignin content or varying the lignin to cellulose composition for easier lignin removal. One report has recently been published describing the downregulation of 4CL in developing xylem cells of aspen (*P. tremuloides*) (Hu *et al.* 1999). A reduction in lignin content of 45% was found, but simultaneously, an increase in cellulose content of 15% and an enhanced growth was observed. A second example given by Lapierre *et al.* (1999) described the downregulation of COMT and CAD in hybrid poplar (*P. tremula x P. alba*). These authors also found a substantial reduction in lignin content together with an increase in cellulose, and a dramatic alteration in lignin structure. Insect resistance has become of a large significance because larvae of some butterflies and other insects are able to defoliate trees. To kill larvae from insects the so-called Bt-toxin from *Bacillus thuringiensis* has been shown to be very effective in tree protection. The gene has been isolated, transferred into the genome of poplar and larch and shown to be active (Klopfenstein *et al.* 1993; Tiang *et al.* 1994). In 35S-*rolC* transgenic hybrid aspen (Fladung *et al.* 1996), it was reported that cell wall components as well as wood structure have been changed. Genetically induced male or female sterility is highly desired when dealing with transgenic trees (Strauss *et al.* 1995).

The basic problem in commercialisation of genetically modified organisms is their very low acceptance in the public. Economic benefits are expected by the industry and horrible risks by the opponents. Transgenic trees can increase economic efficiency of farming and forestry to reduce product costs, can provide important environmental benefits by reducing use of pesticides, and can reduce pressure on native forests for wood, fibre, and energy. On the other hand, however, scientific case-by-case based risk assessment studies are essential which address specific genes and traits but not genetic engineering as a whole. In such risk assessments scientific studies should consider the species which are transformed and the specific ecosystem where the transgenic trees are released, should include symbionts and pathogens, should investigate possible vertical and horizontal gene transfer events, and should include studies on the long-term stability of transgenes. Several research projects on risk assessment of transgenic trees which are currently established in Germany deal with the analysis of the status of phytopathogenic and mycorrhiza fungi in transgenic trees compared to non-transgenic trees. On this level the transgenic plant itself and its communication to other species in the same ecosystem will be considered. An important prerequisite for the long-term use of genetically modified trees is the stable integration and expression of the transgene over a longer period of time. Prerequisite of stable expression of foreign genes is their correct physical integration into the host genome. Using 35S-*rolC* transgenic aspen as a model system (Fladung *et al.* 1997), the expression of the gene and the phenotypical visible reversions have been analyzed (Fladung 1999). It was shown that flanking regions of the transgene should be of non-coding nature and without any AT-rich sequences. Investigations on gene stability are important because trees have long generation cycles, and silencing of foreign genes that is normally not visible can occur at any time during vegetative growth or generative phase. Thus an early selection screen for instability is desired (Tang *et al.* 2002).

Conclusion

The use of these genetically engineered crop hybrids has been widely accepted in the United States but has met opposition elsewhere in the world because of concerns about food safety, environmental protection, and ethical issues. During the past 5 years, the percentage of field corn treated with insecticides in the United States has remained at approximately 30%, despite a significant increase in the hectares of Bt corn planted. A core concept of integrated pest management is to use a management tactic only when pest populations exceed a threshold level. Comparisons of yields from transgenic and genetically similar nontransgenic corn hybrids grown in replicated plots showed that only 34% of the transgenic lines produced significantly higher yields. The use of transgenic crops has been promoted as safer for humans and the environment than use of broad-spectrum insecticides substantial resistance to corn borers. The ecological complexity of agroecosystems needs to be considered. It is urgent to have a moratoium for transgenic insect resistant plant in order to save one of the most valuable biological pesticides. This moratoium is also necessary to prevent genetic pollution via out-crossing. The changed toxin may have the potential to kill others than nontarget organisms that possibly will have far-reaching consequences in different environments. There is now more than evidence that insect-resistant transgenic plants have negative impacts on both sustainable agriculture and the environment. A science-based risk assessment should take into account its own data and not ignore them.

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